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14. ABSTRACT

Previous work in the Hahn Lab has identified *IKBKE* as a novel breast cancer oncogene that is capable of human mammary cell transformation. Amplification and overexpression of IKKepsilon was observed in a significant percentage of human breast cancer cell lines and primary tumor samples. Additionally, breast cancer cell lines carrying the *IKBKE* amplicon showed decreased viability in response to IKKepsilon suppression by shRNA. Our work has also demonstrated that IKKepsilon is a non-canonical IKK (IkappaB kinase) family member that activates the NF-kappaB pathway and that this activity is essential for cell transformation. However, it is known that IKKepsilon not does participate in the canonical IKK complex to activate NF-kappaB signaling. Though recent work has sought to identify the downstream targets of IKKepsilon, the mechanism of its upstream regulation is not well-understood. Thus, I propose to further our understanding of IKKepsilon function by investigating the upstream regulation of IKKepsilon – specifically, the role of ubiquitination in IKKepsilon-mediated cell transformation. In addition, I am to investigate the role of IKKepsilon in breast cancer initiation and maintenance *in vivo* through the generation of a constitutive and an inducible transgenic mouse model.

15. SUBJECT TERMS
breast cancer, ubiquitin, transgenic mouse model, IKKepsilon, NF-kappaB

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Introduction

I have made significant progress in both Specific Aims that were originally laid out in my Statement of Work. In this Progress Report, I will report the studies and results that I have conducted over the past year towards accomplishing those aims.

Specific Aims

1. Investigate the role of ubiquitination in IKKε-mediated cell transformation
    a. Confirm and characterize IKKε ubiquitination in the context of mammary cell transformation
    b. Identification of the IKKε ubiquitin-accepting residues
    c. Determine the functional relevance of IKKε ubiquitination in mammary cell transformation

2. Investigate the role of IKKε in breast cancer initiation and maintenance
    a. Investigate the role of *IKBKE* in breast cancer initiation
    b. Investigate the role of *IKBKE* in breast cancer maintenance

Studies and Results

Specific Aim 1a: I have confirmed that IKKε ubiqitination occurs in a mammary epithelial cell context. I immunoprecipitated IKKε from human mammary epithelial cells that stably overexpress activated MEK (HMECM) along with either Flag-tagged (F-IKKε) or myristolated Flag-tagged (MF-IKKε) IKKε and was able to detect an endogenously ubiquitinated species of MF-IKKε by immunoblotting for ubiquitin (Figure 1A). In parallel, I transiently transfected the same cells with HA-tagged ubiquitin, performed an immunoprecipitation for IKKε and was able to successfully detect an ubiquitinated ladder of MF-IKKε by immunoblotting for HA. I performed the reverse IP/Western experiment, and was also able to immunoblot for IKKε after immunoprecipitation by HA antibody (Figure 1B). Interestingly, I was only able to confirm IKKε ubiquitination in the MF-IKKε transformed HMECM cells and not in the F-IKKε transformed cells. Previous characterization of these cell lines has shown that MF-IKKε exhibits a much more robust transformation phenotype in HMECM cells than the F-IKKε counterpart. Perhaps this variation in transformation phenotype is related to the ubiquitination status of IKKε in these cells.

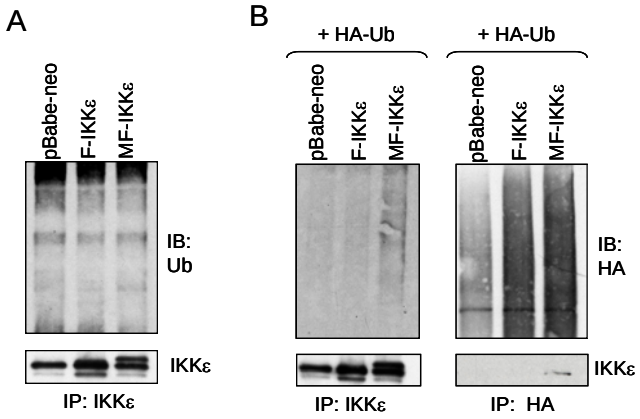
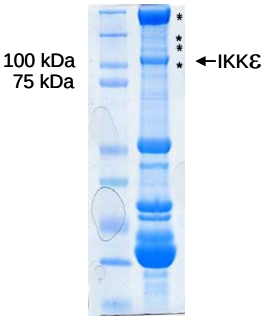


Figure 1. IKKε is ubiquitinated HMECM cells.
(A) Lysates from HMECM cells expressing either pBn, F-IKKε or MF-IKKε. were immunoprecipitated by the IKKε antibody and immunoblotted for ubiquitin.
(B) HMECM F-IKKε or MF-IKKε. cells were transfected with HA-Ub. Lysates were immunoprecipitated by IKKε or HA antibody and then immunoblotted for the other protein.

I have attempted to characterize the lysine linkage of IKKε using K48- or K63- only ubiquitin mutants. However, I have thus far found that these ubiquitin mutants have yielded inconclusive immunoprecipitation and immunoblot results. This has made it difficult to specifically characterize the nature of the interaction. I am currently working on optimizing the conditions for the use of these ubiquitin mutant constructs.

Specific Aim 1b: I have successfully identified three lysine residues in IKKε that are subject to ubiquitination by mass spectrometry. I transiently cotransfected GST-tagged IKKε and HA-tagged ubiquitin in HEK293T cells and performed a GST immunoprecipitation. The immunoprecipitates were then subjected to SDS-PAGE followed by Coomassie blue staining. Four bands of interest were identified and submitted for mass spectrometry analysis (Figure 2). We obtained 58.2% coverage of the IKKε protein and 64.7% (22 out of 34) coverage of the internal lysines. From this analysis, three lysine residues were identified as modified by ubiquitin: K30, K401, and K416.



1	MQSTANYLWH	TDDLLGQGAT	ASVY**K**ARN**KK**	SGELVAV**K**VF	NTTSYLRPRE
51	VQVREFEVLR	**K**LNHQNIV**K**L	FAVEETGGSR	Q**K**VLVMEYCS	SGSLLSVLES
101	PENAFGLPED	EFLVVLRCVV	AGMNHLRENG	IVHRDI**K**PGN	IMRLVGEEGQ
151	SIY**K**LTDFGA	ARELDDDE**K**F	VSVYGTEEYL	HPDMYERAVL	R**K**PQQ**K**AFGV
201	TVDLWSIGVT	LYHAATGSLP	FIPFGGPRRN	**K**EIMYRITTE	**K**PAGAIAGAQ
251	RRENGPLEWS	YTLPITCQLS	LGLQSQLVPI	LANILEVEQA	**K**CWGFDQFFA
301	ETSDILQRVV	VHVFSLSQAV	LHHIYIHAHN	TIAIFQEAVH	**K**QTSVAPRHQ
351	EYLFEGHLCV	LEPSVSAQHI	AHTTASSPLT	LFSTAIP**K**GL	AFRDPALDVP
401	**K**FVP**K**VDLQA	DYNTA**K**GVLG	AGYQALRLAR	ALLDGQELMF	RGLHWVMEVL
451	QATCRRTLEV	ARTSLLYLSS	SLGTERFSSV	AGTPEIQEL**K**	AAAELRSRLR
501	TLAEVLSRCS	QNITETQESL	SSLNRELV**K**S	RDQVHEDRSI	QQIQCCLD**K**M
551	NFIY**K**QF**KK**S	RMRPGLGYNE	EQIH**K**LD**K**VN	FSHLA**K**RLLQ	VFQEECVQ**K**Y
601	QASLVTHG**K**R	MRVVHETRNH	LRLVGCSVAA	CNTEAQGVQE	SLS**K**LLEELS
651	HQLLQDRA**K**G	AQASPPPIAP	YPSPTR**K**DLL	LHMQELCEGM	**K**LLASDLLDN
701	NRIIERLNRV	PAPPDV			

Figure 2. Mass spectrometry of IKKε.
(Left) GST-IKKε and HA-ubiquitin (HA-Ub) were coexpressed in HEK293T cells and lysates from 45 x 10 cm plates were loaded on SDS-PAGE and stained with Coomassie Blue. The bands noted by asterisks were submitted for mass spectrometry (Right) Summary of mass spectrometry data. Underlined amino acids were identified by mass spectrometry. Lysines (K) are bolded and the three lysines in red were found to be ubiquitinated.

Specific Aim 1c: I have successfully generated site-specific lysine-to-alanine IKKε mutants for the three lysine residues that were identified in Aim 1b. All possible combinations of single, double and triple mutants of the protein were generated and then retrovirally introduced into HA1EM cell to create stable cell lines that express these mutant constructs. These mutants were assessed for transformation capacity by soft agar analysis (Figure 3). From this analysis, I have determined that the K30 and K401 residues are essential for IKKε-mediated transformation. However mutation of the K416 residue does not perturb the transformation phenotype of IKKε. Surprisingly, in addition, I have observed that the mutation of the K416 residue rescues the decreased transformation phenotype of the K30 and K401 mutants. This may be due to a

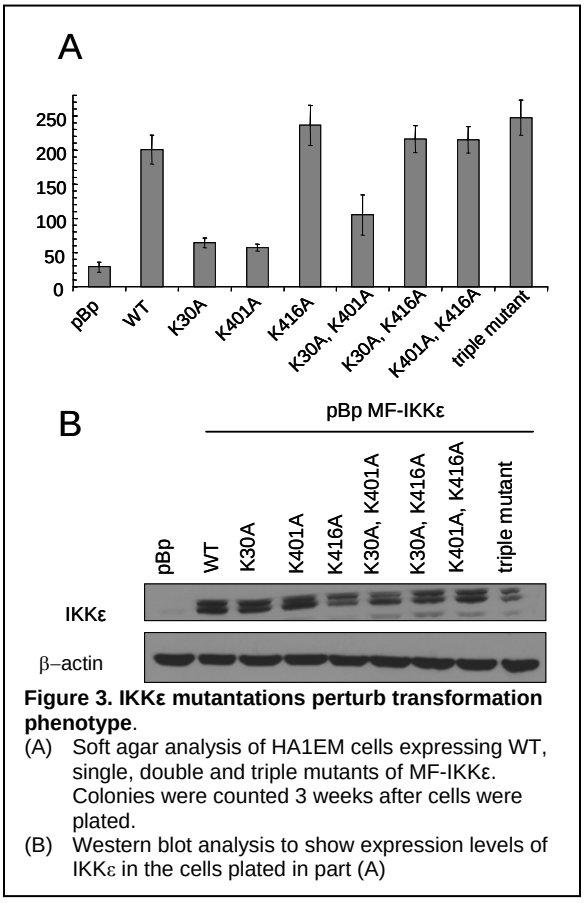


Figure 3. IKKε mutantations perturb transformation phenotype.
(A) Soft agar analysis of HA1EM cells expressing WT, single, double and triple mutants of MF-IKKε. Colonies were counted 3 weeks after cells were plated.
(B) Western blot analysis to show expression levels of IKKε in the cells plated in part (A)

combination of positive and negative regulation of IKKε by both Lysine48- and Lysine63- linked ubiquitination.

In order to assess the tumorigenicity capacity of these mutants, all mutant IKKε cell lines have been injected into immunocompromised mice (6 injections per cell line) and are currently being observed for tumor formation.

Specific Aim 2a: I have successfully generated a MMTV-IKKε transgenic mouse model. By genotyping analysis, 9 founder mice were identified. These founders were bred out and assessed for mammary gland-specific expression of IKKε by immunoblot (Figure 4). From this analysis, 3 founder lines with varying levels of IKKε expression were chosen and are currently being expanded and followed. The original founders are now ~8 months old and have not shown a mammary tumor formation phenotype.

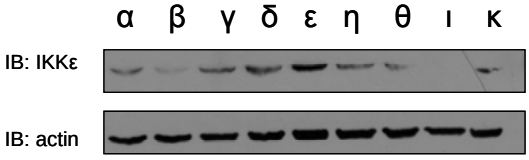


Figure 4. Analysis of MMTV-IKKε founder lines. 9 IKKε founder lines were established and analyzed for mammary gland specific IKKε expression by Western blot. IKKε expression is assessed by the upper band in the Western analysis.

In addition, these founder lines are also being bred to MMTV-ErbB2 mice, which are known to have a delayed mammary gland tumor formation phenotype. These MMTV-IKKε/ErbB2 bitransgenic mice will allow us to determine if there is a synergistic tumor formation phenotype, should the MMTV-IKKε mice not develop tumors on their own. We have also obtained WAP-Cre and p53 floxed mice from the Mouse Models of Human Cancer Consortium (MMHCC) and are prepared to cross the MMTV-IKKε mice into a mammary-specific p53 null background, if needed.

Specific Aim 2b: We have successfully generated a doxycycline-inducible MMTV-rtTA/TetO-IKKε bitransgenic mouse model. By genotyping analysis, 5 founder mice were identified (Figure 5). After analysis for doxycycline-inducible mammary gland IKKε expression, 2 founder lines with varying levels of IKKε expression were chosen and are currently being expanded and followed.

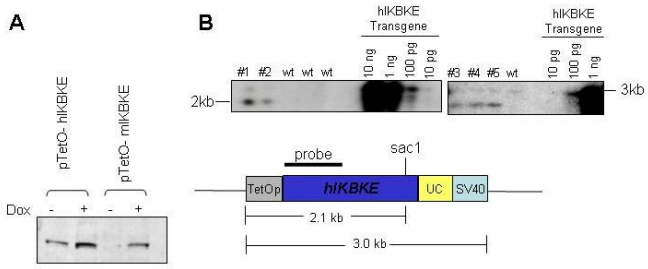


Figure 5. Inducible *hIKBKE* transgenic founder mice established
(A) NIH3T3 cells that stably express rtTA are transfected with the inducible transgenic construct pTetO-hIKBKE (human cDNA) and pTetO-mIKBKE (mouse cDNA). Cells were treated with 10μg/ml doxycycline for 24 hours. Immunoblot analysis (WB) on lysates was performed with an IKKε antibody
(B) Genomic DNA was made from tails of putative transgenic mouse pups and digested with SacI. Southern genotyping analysis was performed using a probe that hybridizes to the 3' region of the human *IKBKE* transgene. Linearized hIKBKE transgene was used at varying DNA concentrations as a control. Five founder mice were genotyped and contain the hIKBKE transgene in varying copy numbers.

Key Research Accomplishments

- Confirmed IKKε ubiquitination in the context of mammary cell transformation
- Identified three lysine residues within IKKε that are subject to ubiquitination by mass spectrometry
- Generated IKKε point mutants in which the identified lysine residues are mutated singularly and in all possible tandem combinations
- Generated cell lines that stably express the IKKε mutant constructs
- Demonstrated that IKKε lysine mutants show a distinct change in transformation phenotype *in vitro*
- Generated founder mice for the constitutive MMTV-IKKε transgenic mouse model
- Generated founder mice for the inducible MMTV-rtTA/TetO-IKKε transgenic mouse model

Reportable Outcomes

- Developed 14 HA1EM mutant IKKε cell lines: both F-IKKε and MF-IKKε with the following mutations K30A; K401A; K416A; K30A K401A; K30A K416A; K401A K416A; K30A K401A K416A
- Developed two new animal models: MMTV-IKKε and MMTV-rtTA/TetO-IKKε

Conclusion

Over the past year, I have made significant progress in all aspects of my Specific Aims as laid out in my original Statement of Work.

I have been able to identify three residues of IKKε that undergo modification by ubiquitination, and I have been able to show that the mutation of these residues is able to perturb the IKKε-mediated transformation phenotype. However, my mutational studies have also indicated that there is a more complex and dynamic role for IKKε ubiquitination than I had originally hypothesized. I believe that IKKε is undergoing a combination of both Lys48- and Lys63- linked ubiquitination, and that this combination of modifications is serving to tightly regulate both the levels of IKKε protein in the cell as well as its enzymatic activation. I propose to do a series of IP/Western experiments that utilize the Lys48- and Lys63- only ubiquitin mutants in combination with the IKKε mutants, in order to determine the exact nature of these modifications. In addition, to understand how the perturbation of the various residues of IKKε affects its enzymatic activity, I propose to perform *in vitro* kinase assays to assess the enzymatic activity of the various IKKε mutants. Finally, I will also assay for NF-κB activation in these mutant cells to determine how the perturbation of IKKε ubiquitination affects its role in this pathway.

I have also successfully generated both a constitutive and inducible transgenic mouse model of mammary-specific IKKε expression. As originally laid out in my Statement of Work, I am currently working to generate a cohort of 50 mice to assess for a mammary tumor formation phenotype. In parallel, I have bred the constitutive MMTV-IKKε with MMTV-ErbB2 mice to determine if there is a synergistic tumor formation phenotype, should the MMTV-IKKε mice not develop

tumors on their own. In addition, we also have WAP-Cre and p53 floxed mice and are prepared to cross the MMTV-IKKε mice into a mammary-specific p53 null background, if needed.